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Review

# Ion-pair reversed-phase chromatography of short double-stranded deoxyribonucleic acid in silicon micro-pillar array columns: Retention model and applications

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ABSTRACT

Separation of double-stranded (ds) DNAs is important in numerous biochemical analyses relevant for clinical applications. A widely used separation technique is high performance liquid chromatography (HPLC), in the variant of ion-pair reversed-phase (IP-RP) chromatography. HPLC can be miniaturized by means of silicon micro-pillar array columns leading to on-chip fast and high resolution dsDNA separation with limited sample quantity. However, theoretical studies of retentive behavior of dsDNA in miniaturized chromatographic columns are hardly available, despite their enormous practical relevance. This paper established a new retention model to describe the size dependent separation of dsDNAs for any characteristic of the linear mobile phase gradient, in analogy to the model used to describe the retention of polymer chains with repeating units in RP HPLC. The model agrees with a large amount of dsDNA retention data, measured using DNA molecules in the size range of 10–400 base pairs in columns with different lengths (2 and 40 cm) and different micro-pillar sizes (2 and 2.5 μm in diameter), in various mobile phase gradients. The model is particularly useful in practice, since it requires no numerical solutions and the column-specific fitting parameters (4 or 5) can be determined in a limited number of separation runs. As examples of its applications, the model has been used for the optimization of dsDNA step-gradient separations (5 dsDNAs separated within 8 min) and for the determination of the size of dsDNA fragment (with uncertainty of about 2%). These applications are especially relevant for on-chip DNA analysis devices.


**Contents**



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## 1. Introduction

Microfabricated separation devices provide a powerful approach to miniaturization and integration of analysis systems, which have abundant applications in life science research, clinical diagnostics, drug discovery and biotechnology. Nucleic acid separation is considered as one of such important applications, holding promise to enable fast and easy detection of DNA related diseases on portable systems. HPLC in IP-RP chromatography mode is very successful for nucleic acid separation and the best results are obtained using polystyrene divinylbenzene (PS-DVB) columns [1–4]. Little attention has been dedicated until now to nucleic acid chromatography in miniaturized systems. Only recently, a LC chip constructed of a polyimide substrate became commercially available by Agilent [5]. The polyimide serves here as container wherein particles are packed, just like in a traditional packed bed column in a disordered fashion. In a more extended miniaturization approach, the support structures are directly fabricated, in an ordered fashion, with sizes down to a few hundreds of nanometers [6–9]. In this approach, silicon is superior to the polymer substrates commonly used in microfluidic devices. The grafting possibilities that deep reactive ion etching protocols offer with silicon substrates are unmatched compared to other substrates and the best available processing methods. Therefore, several important benefits of performing chromatography in micro-pillar arrays, owing to the use of advanced silicon fabrication technology, can be listed: the ordered nature of the pillar array results in a reduction of the theoretical plate height by a factor of two compared to a packed column with equally sized particles [8]; the shape and positioning of pillars can be tailored with a high degree of freedom, allowing the conception of dedicated columns for individual applications; in this micro-pillar format, the 'column' is only 2–4 cm long and a flow of 1 μl/min generates a pressure drop of only a few hundreds of kPa, which can be provided by on-chip pumps [10]; a small quantity of sample is required in the micro-pillar array column, which is beneficial when only a minute amount of analyte is available, and which also has a positive impact on the overall separation resolution and efficiency.

Nucleic acid HPLC offers a comparable performance as capillary electrophoresis (CE), the most used nucleic acid separation technique. Using conventional PS-DVB HPLC columns, the resolution of DNA fragments smaller than 1000 base pairs (bp) is about 2–8% of their length and the analysis time ranges from less than 1 min to 30 min [2]. These values are very competitive to those obtained with CE [11]. Dealing with analyte, HPLC requires little or no prior sample preparation or purification, an advantage that it shares with CE [12]. In LC systems the nucleic acid can be recovered after separation by evaporating the solvent [2], a similar approach can probably be used in CE, but the solvent is less volatile. Additionally, an interesting feature of the presented separation method is the ability to immobilize and selectively elute a specific sized DNA fragment by controlling the mobile phase. This behavior is clearly different from that occurring in CE, where all components migrate during the entire period of a separation experiment. This feature can be a possible route toward a control of the timing of several events when aiming at an integration of several operations like chemical derivatization, digestion, detection, etc. in a so-called lab-on-a-chip device. Considering all these aspects of HPLC in nucleic acid separation, we are convinced that it is very attractive to study DNA chromatography on chip.

Among many HPLC techniques, IP-RP chromatography, along with ion-exchange chromatography (IEC), is a highly suitable technique for DNA separations because of its excellent resolution and ability to separate mixtures containing long fragments of both single and double-stranded polynucleotides. In contrast to IEC, where the base paired sequence exerts a complex influence on retention [2], the elution order of dsDNA in IP-RP chromatography reflects the size of the molecules in a very straightforward way. Retention is predominantly controlled by the overall charge and hence by the number of the nucleotides in the polynucleotide chain that form ion-pairs with amphiphilic cations in mobile phase, and only marginally by the base composition [2]. Therefore we have chosen IP-RP chromatography to perform dsDNA separation on silicon micro-pillar chips.

Generally, the chromatographic separation of large biomolecules (which we consider as having molecular weights larger than 10,000 Da) is a complex process [13,14]. We earlier observed an "on–off" type of interaction between dsDNA molecules and micro-pillar surface [15], which makes isocratic separation mode unpractical, hence necessitating the implementation of a gradient system. The use of this separation technique on chip usually requires the ability to predict and control retention, for analytical as well as for preparative separations. It is hence crucial to establish a model which can explain and predict the retention behavior of dsDNA molecules. So far only empirical models were used to describe the DNA size dependence of retention in IP-RP chromatography. In 1995, Huber et al. presented the retention capacity factor as a function of the logarithm of the number of DNA base pairs [3]. Later in 2002, Gilar et al. introduced a model where the concentration of the organic modifier necessary for eluting a given fragment is expressed in terms of the logarithm of dsDNA base pairs [4]. Although these models provide good predictions for the size dependence of retention, the fitting-parameters do not have a clear physical meaning and their values depend on the choice of mobile phase, and hence have to be determined again when the mobile phase characteristics are changed. A good theoretical model for dsDNA IP-RP chromatography needs to consider two particular aspects: the large size of dsDNA molecule, which behave differently from commonly used small molecular compounds (molecular weights less than 10,000 Da), and the role of IP in generating retention, which is absent in standard RP chromatography. The RP chromatographic separation of many large molecules, such as peptides, proteins [14,16] and poly(ethylene glycol)s [17] and oligonucelotides [18,19], has been studied theoretically widely. The models are based on the conventional gradient theory developed for small molecular compounds, a work published as early as 1978 by Schoenmakers et al. [20]. Since 1984, Jandera et al. have studied the retention behavior of homologous series [21], oligomeric series [22] and synthetic (co)polymers [23] with respect to the number of repeat structural units in RP chromatography. Meanwhile, many theoretical models [24,25] were dedicated to explain the physical foundation of IP-RP chromatography. Important contributions, based on electrostatic theories, were presented by Cantwell and co-workers [24] and by Ståhlberg and co-workers [26]. All the theories developed so far studied the retention behaviors of small molecular compounds intensively but did not address the retention behavior of large molecules. Furthermore, one needs to be aware that these theories for IP-RP chromatography are based on two different points of views, none of which is completely correct, as will be discussed in detail in the theory section below.

To go beyond existing models, we established a practical model which includes the impact of both column properties and experimental variables on retention behaviors and which is able to describe IP-RP chromatographic retention behavior of dsDNA molecules with respect to the number of base pairs in the micro-pillar array columns. To confirm the validity of the model, we separated dsDNA samples with a size range of 10–400 bp in columns with different lengths (2 and 40 cm) and composed of different micro-pillar configurations (2 and 2.5 μm in diameter) using various mobile phase conditions. Two applications were